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ORIGINAL ARTICLE

Immune cell and cytokine patterns in children with type 1 diabetes mellitus undergoing a remission phase: A longitudinal study

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Objective: Type 1 diabetes (T1D) develops in distinct stages, before and after disease onset. Whether the natural course translates into different immunologic patterns is still uncertain. This study aimed at identifying peripheral immune patterns at key time-points, in T1D children undergoing remission phase.

Methods: Children with new-onset T1D and healthy age and gender-matched controls were recruited at a pediatric hospital. Peripheral blood samples were evaluated by flow cytometry at 3 longitudinal time-points: onset (T1), remission phase (T2) and established disease (T3). Cytokine levels were quantified by multiplex assay. Fasting C-peptide, HbA1c, and 25OHD were also measured.

Results: T1D children (n = 28; 10.0 ± 2.6 years) showed significant differences from controls in circulating neutrophils, T helper (Th)17 and natural killer (NK) cells, with relevant variations during disease progression. At onset, neutrophils, NK, Th17 and T cytotoxic (Tc)17 cells were decreased. As disease progressed, neutrophil counts recovered whereas NK counts remained low. Th17 and Tc17 cells behavior followed the neutrophil variation pattern. B-cells were lowest in the remission phase and regulatory T-cells significantly declined after remission. Two cytokine response profiles were identified. Low cytokine-responders showed higher circulating fasting C-peptide levels at onset and longer remission periods. C-peptide inversely correlated with pro-inflammatory and cytotoxic cells.

Conclusions: Our data suggest an association between immune cells, cytokine patterns and metabolic counterparts. The dynamic changes of circulating immune cells during disease progression involve key innate and acquired immune cell types. This longitudinal picture of T1D progression may enable disease staging and patient stratification, essential for individualized treatment.

KEYWORDS

c-peptide, cytokines, diabetes mellitus, type 1, lymphocytes, neutrophils

1 | INTRODUCTION

Type 1 diabetes (T1D) is a metabolic disorder in which the interaction between genetic susceptibility, environmental factors and immunologic mechanisms leads to a selective destruction of pancreatic insulin-producing beta-cells.[1] This chronic and life-debilitating disease affects a growing number of young children,[2] yet its etiology and pathogenesis are still unresolved. A T effector/T regulatory (Treg) cell imbalance has been considered, allowing the expansion of cytotoxic $CD8^+$ T-[3] and B-cells,[4] as observed in animal models, along with autoantibody production. T1D is also related to MHC-gene polymorphisms[5] implying an involvement of the adaptive immune system.

Nevertheless, increasing evidence suggests that innate immune cells and non-specific inflammation also play a critical role in the pathogenesis of T1D, as shown by studies in pancreatic insulitis and peripancreatic ganglia.[6,7]

Along the natural history of T1D, periods of immunologic tolerance and autoimmune activity occur, probably as a consequence of antigen determinants spreading, beta-cell proliferation or cytotoxic T-cell action.[8]

It is well recognized that in 40% to 71% of children,[9,10] T1D develops in distinct phases, corresponding to different metabolic and possibly immunological stages.[8] After onset, a remission phase or "honeymoon" may occur, with a partial and transitory restoration of endogenous insulin production. This natural phenomenon has been attributed to lower metabolic glucotoxicity, due to action of exogenous insulin and glycaemic improvement. Several authors have hypothesized that the "honeymoon" is a phase of immunotolerance in a relapse-remitting disease.[8,11]

Studies approaching peripheral blood (PB) and target organ are crucial to identify biomarkers as predictors for early and individualized intervention. Moreover, the dynamics of disease course in humans requires a thorough investigation to identify an optimal therapeutic window for induction of beta-cell regeneration and restoration of immunotolerance.

Current knowledge of T1D pathogenesis relies mostly on animal models, which do not fully mirror the human T1D pathology, as recently documented in a direct comparative study.[12] On the other hand, the risks of presently available techniques to access the pancreas in vivo restrain organ studies in humans, and consequently human data on disease progression are rare.[13,14]

Circulating immune cells in T1D patients may reflect the events occurring in the pancreas[15] and may predict the occurrence of the remission phase,[16] but prospective longitudinal data are limited. One study in children with T1D suggested that specific immunological signatures are related to disease severity[17] and functional changes in circulating Treg have been reported at different disease stages.[18]

Thus, we hypothesize that changes in the profile of PB immune cell subsets and cytokines may reflect the natural course of T1D in humans. This study aimed to identify peripheral immune patterns in T1D children during disease progression, and to determine whether a link between the inflammatory status and metabolic severity exists. The longitudinal picture of T1D progression may enable disease staging and patient stratification, which will allow for individualized treatment.

2 | METHODS

2.1 | Study population

Children with new-onset T1D were recruited at admission for 18 months in a central pediatric hospital. Inclusion criteria were: age 5 to 17 years old, and <14 days after diagnosis. T1D was defined according to the ADA criteria,[19] with at least one positive autoantibody (anti-insulin; Insulinoma-Associated-2; antiglutamic acid decarboxylase). Children with other autoimmune diseases, allergy or acute infections were excluded. Controls (age and gender-matched) were selected from the pediatric endocrinology outpatient clinic, where they were evaluated due to variants of normal growth and/or puberty. Initially, 42 patients were recruited, of which 28 completed the study (Figure 1).

2.2 | Study design

Blood samples were collected at 3 distinct time-points throughout individual disease development. At disease onset (time-point 1: T1), samples were collected between 1 and 14 days after diagnosis. At partial remission (time-point 2: T2) and at "established disease" (time-point 3: T3), samples were collected according to the value of Insulin dose adjusted to HbA_{1c} ($IDAA_{1c}$), used as a surrogate measure of endogenous insulin production. $IDAA_{1C} = HbA_{1C}(\%) + (4\times$ insulin dose [units per kilogram per 24 hours]).[20] T2 was collected once the patient entered partial remission phase ($IDAA_{1C} \leq 9$) and T3 was collected once patients entered established disease phase, that is, $IDAA_{1C} > 9$.

Control samples were collected simultaneously to T1. Longitudinal data collection occurred in a 2-year time-period. The number of cases admitted to the hospital during the study period determined the initial sample size. For study evaluation, only patients with successful sample collection in all time-points were considered (Figure 1).

The study was approved by the hospital's ethics committee and conducted in accordance with the Declaration of Helsinki. Informed consent forms were signed.

2.3 | Sample collection

Blood samples for immunophenotyping and cell stimulation were collected in EDTA and heparin tubes, respectively, and processed within 24 hours. For cytokine quantification, sera were obtained by centrifugation after clot retraction, separated in aliquots and stored at −20°C until analysis.

2.4 | Flow cytometry

Multicolor flow cytometry was used for characterization of immune cell subsets, using a BD FACS Calibur (BD Biosciences, San Jose, California) equipped with 2 lasers (488 nm air-cooled argon-ion laser, and 635 nm red diode laser). For characterization of leukocyte and lymphocyte subpopulations the BD Multitest IMK Kit with Trucount absolute counting tubes (BD Biosciences) was used, according to manufacturer's instructions.

For Treg phenotyping, the panel of mAbs included anti-CD3-FITC (clone SK7, BD Biosciences), anti-CD25 PE (clone BC96, Biolegend, California), anti-CD4 PerCPCy5.5 (clone SK3, Biolegend) and anti-CD127 AlexaFluor647 (clone A019D5, Biolegend) (Additional information in Figure S1, Supporting Information).[21,22]

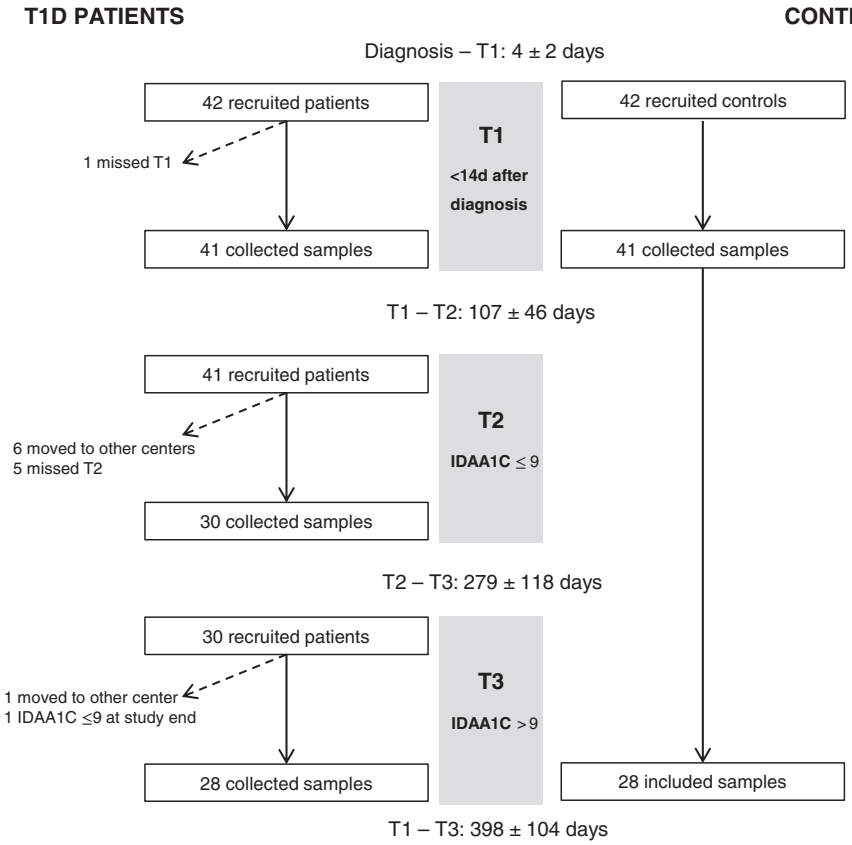

FIGURE 1 Study design.
T1D, type 1 diabetes;
T1, time-point 1 (new-onset disease);
T2, time-point 2 (remission phase);
T3, time-point 3 (established disease);
$IDAA_{1C}$, insulin dose adjusted HbA_{1C}

2.5 | Cell stimulation for intracellular cytokine evaluation

The expression of IFN-γ and IL17 was used to assess Th1 and Th17 cells. For this, heparinized whole blood samples were stimulated with PMA (50 ng/mL; Sigma Aldrich, St. Louis, Missouri) and calcium ionophore (1 μg/mL, Sigma Aldrich) for 5 hours at 37°C in a 5% CO_2 atmosphere, in the presence of Brefeldin A (1.0 μg/mL, BD Pharmingen, San Jose, California). For control purposes, unstimulated cells were incubated in parallel. The protocol was performed according to the Cytofix-Cytoperm kit (BD Pharmingen) instructions (Additional information in Figure S2).

2.6 | Serum cytokines quantification by Luminex technology

Serum cytokines (IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IL17-A, IFN-γ, MCP-1 and TNF-α) were accessed by a multiplex strategy. A 10-plex Bioplex Human Cytokine Assay kit (Bio-Rad Laboratories, Inc., Hercules, California) was used for the quantification, according to manufacturer recommendations.

2.7 | Indicators of metabolic status

Glycated hemoglobin HbA_{1C} was determined by chromatography (Variance 2-Biorad, Hercules, California) and fasting C-peptide and 25-OHD were determined by chemiluminescence (Immulite 2000, Siemens, Germany).

2.8 | Statistical analysis

GraphPad Prism software v.6.01 (GraphPad Software, La Jolla, California) was applied. Categorical variables were analyzed by Fisher's exact test or χ^2. Normality of distributions was assessed by D'Agostino & Pearson test. Data normally distributed were presented as mean ± SD, otherwise as median and interquartile range. Paired groups were analyzed with paired Student's *t* test or Wilcoxon test. Unpaired Student's t or Mann-Whitney tests were used to compare each 2 independent groups. $P < .05$ was considered significant.

3 | RESULTS

3.1 | Characteristics of the study population

Table 1 summarizes demographic and metabolic data from T1D patients and controls. The T1D group comprised 28 children (mean age: 10.0 ± 2.6 years, 46% males). At disease onset (T1), samples were collected 4 ± 2 days after diagnosis. At partial remission (T2), average sample collection time was 107±46 days after diagnosis, and at established disease (T3) 398±104 days after diagnosis. Remission phase (T2-T3) duration was on average 279 ± 118 days (Figure 1). No relevant seasonal clustering was identified.

3.2 | Neutrophils and NK-cells were decreased at T1D onset with regain of neutrophils but not of NK-cells

Compared to controls, both neutrophils and NK-cell counts were significantly decreased in TD1 children at disease onset ($P = .0439$;

TABLE 1 Characteristics of the study population

	Age, years (mean, SD)	Sex (M/F)	Onset BMI, kg/m^2 (mean, SD)	Positive AIA	Positive IA2	Positive GAD	Onset HbA$_{1c}$, % (mean, SD)	Onset fasting C-peptide, ng/mL (median, IQR)
Patients (n = 28)	10 (3)	14/14	16.8 (3.0)	24/28	9/28	10/28	12.7 (2.4)	0.3 (0.2; 0.6)
Controls (n = 28)	10 (3)	14/14	—	—	—	—	—	—

Abbreviations: AIA, anti-insulin autoantibody; F, female; GAD, antiglutamic acid decarboxylase autoantibody; IA2, insulinoma-associated-2 autoantibody; IQR, interquartile range; M, male; SD, standard deviation.

P = .0115, respectively) (Figure 2B,D). The frequency of NK-cells was also significantly lower (P = .0012) (Figure 2C). Upon disease progression, neutrophils increased, both in absolute counts (T1-T2 P = .1375; T1-T3 P = .0082; T2-T3 P = .0112) and frequency (T1-T2 P = .0564; T1-T3 P = .0005; T2-T3 P = .0089). This neutrophil recovery was most pronounced at the final stage, reaching the control group values (Figure 2A,B). Similarly, NK-cells significantly increased during disease progression (T1vsT2 P = .0139; T1vsT3 P = .0188; T2vsT3 P = .9670). However, absolute counts and percentages remained diminished at T3 (respectively, P = .0307; P = .0789) compared to controls (Figure 2C,D).

3.3 | B-cells reached their lowest level during the remission phase

B-cells significantly decreased from T1 to T2, without further changes, both in absolute number (T1-T2 P = .0002; T1-T3 P = .0006) and frequency (T1-T2 P = .0003; T1-T3 P = .0075) (Figure 2E,F). No correlation was identified for B-cells, considering metabolic parameters (C-peptide, HbA1c, and vitamin D) and duration of disease stages.

3.4 | The regain of pro-inflammatory Th1 and Th17 cells after T1D onset was accompanied by a decline of regulatory T-cells after remission

Compared to control values, Th1 and Tc1 cell subsets did not show significant differences in T1D children at any time-point. However, a significant increase was detected during the disease course (Th1% T1-T2 P = .0041, T1-T3 P = .0014; Tc1% T1-T2 P = .0032, T1-T3 P = .0004). The rise of these pro-inflammatory cells occurred mainly in stage T2 (Figure 3A,B). Differently, Th17 and Tc17 cell populations were significantly decreased, in T1D children compared to controls, already at disease onset (Th17% P = .0047, Th17 cells/μL P = .0155; Tc17% P = .0003, Tc17 cells/μL P = .0017). Although there was a significant increase during disease progression (Th17% T1-T2 P = .0322; T1-T3 P = .0207; Tc17% T1-T3 P = .0218), both populations remained below control levels (Figure 3C,D). Treg frequency and numbers were not significantly different from controls at any of the 3 time-points. However, Treg significantly decreased in the established disease phase (Treg% T2-T3 P = .0016; Treg cells/μL T1-T3 P = .0028; T2-T3 P = .0497) (Figure 3E,F). Addressing the ratios, Th17/Treg was significantly lower at disease onset, compared to controls (P = .0117). Th1/Treg was not different at any time-point. Along the disease course, both ratios significantly increased (Th1/Treg T1-T3 P = .0036; Th17/Treg T1-T3 P = .0118).

3.5 | C-peptide at T1D onset was related to the immune cell pattern and duration of the remission phase

At disease onset, C-peptide inversely correlated with absolute counts of pro-inflammatory and cytotoxic cells, namely T-cells (r = −.395; P = .042) (Figure 4A), NK-cells (r = −0.486; P = .010) (Figure 4B), Tc1 cells (r = −0.479; P = .011) (Figure 4C) and total IFN-γ-secreting CD8 T-cells (r = −0.481; P = .011). A positive correlation between C-peptide at disease onset and remission duration was also identified (r = 0.381; P = .050). Children with lower C-peptide at onset had shorter remission phases.

3.6 | Limited impact of vitamin D status at T1D onset

To address the effect of seasonality, we compared T1D patients with disease onset from November to April with patients diagnosed from May to October. Our results revealed no significant differences regarding vitamin D levels and immune cells and cytokines in the 2 groups. Also, at T1D onset, vitamin D levels did not correlate with any of the immune parameters evaluated (cell subsets and cytokines).

3.7 | Serum cytokines at disease onset were related to C-peptide levels and duration of the remission phase

Regarding cytokine concentrations, no differences were found for any studied cytokines between T1D children (T1, T2, or T3) and controls; neither significant variation along disease progression was observed.

However, we identified 2 cytokine patterns within our patients: low responders (LR), with no detectable anti-inflammatory cytokines (IL-4, IL-10, IL-13) and no pro-inflammatory TNF-α; high responders (HR) with at least one of these cytokines detected (IL-4, IL-10, IL-13, or TNF-α). Consistently, the HR group presented higher levels of other cytokines such as IL2 (P = .038), IL-6 (P = .002), IFN-γ (P < .001) and MCP-1 (P = .004) at onset; IL2 (P = .014), IL-17A (P = .022) and IFN-γ (P = .046) at partial remission and IL1-β (P = .019), IL2 (P = .003), IL-6 (P = .004), IL-17A (P = .035), IFN-γ (P = .002) and MCP-1 (P = .009) after disease establishment.

LR had both higher circulating fasting C-peptide levels at disease onset (P = .008) and longer remission periods (P = .005) compared to HR (Figure 5A,B). There were no differences between LR and HR regarding immune cell subsets. No correlation between cytokine levels and age was identified.

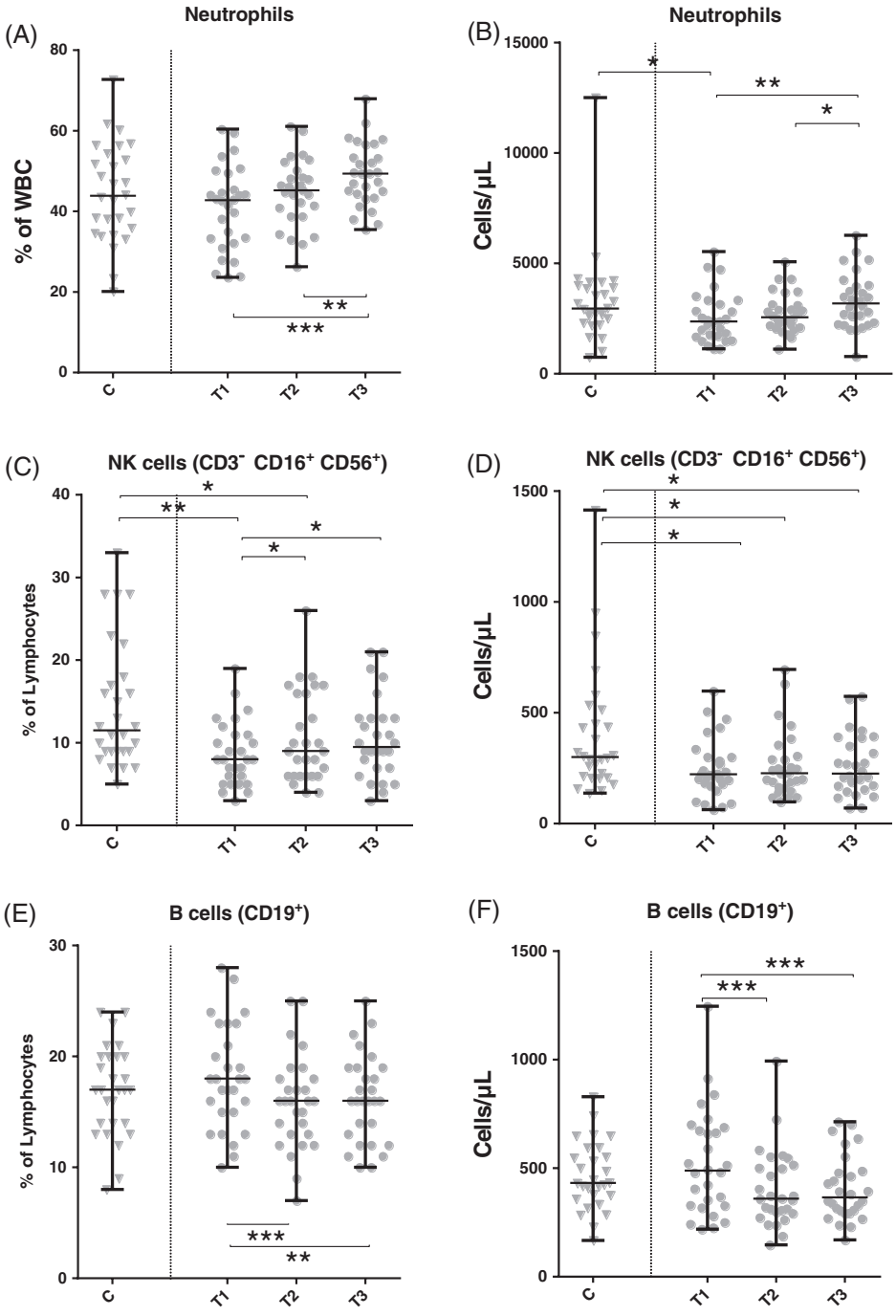

FIGURE 2 Neutrophils, NK and B-cells peripheral immune pattern from T1D onset to established disease. Percentages and absolute counts of peripheral neutrophils (A, B), NK (C, D) and B-cells (E, F) from T1D children and healthy controls. PB collection at disease onset (T1), partial remission (T2) and disease establishment (T3). *P < .05, **P < .01, ***P < .001 by Mann-Whitney test (patients vs controls) or Wilcoxon matched-pairs signed rank test (comparison within patients in different time-points)

4 | DISCUSSION

Various models of T1D disease progression have been published, based mostly on animal studies.[12] To the best of our knowledge, this is the first study which prospectively monitors immune cell subsets and cytokines in T1D children from disease onset throughout the remission phase up to disease final establishment.

T1D children differed significantly from healthy controls in circulating neutrophils, Th17 and NK-cells, with relevant variations at onset, partial remission and disease establishment. In addition, B-cells, Treg and IFN-γ producing T-cells significantly varied during disease progression. The natural history of T1D seems to be modulated by immune parameters, as they correlate with residual insulin production. Considering the cytokine environment, 2 profiles were identified in T1D patients, low and high responders, with significantly different basal C-peptide levels at disease onset as well as distinct periods of remission.

The neutrophil reduction at disease onset has previously been described in humans with T1D but was not evident in T2D patients, suggesting that such a decrease is not due to impaired glycaemic control.[23] The reason for decreased neutrophil counts is most likely tissue sequestration. Accordingly, data on T1D pancreas morphology have shown neutrophil infiltration in exocrine pancreas at disease onset.[23,24] Recruitment of these innate immune cells to the pancreas by the pro-inflammatory IL-17 environment is one of the proposed mechanisms by which neutrophils participate in T1D.[7] Furthermore,

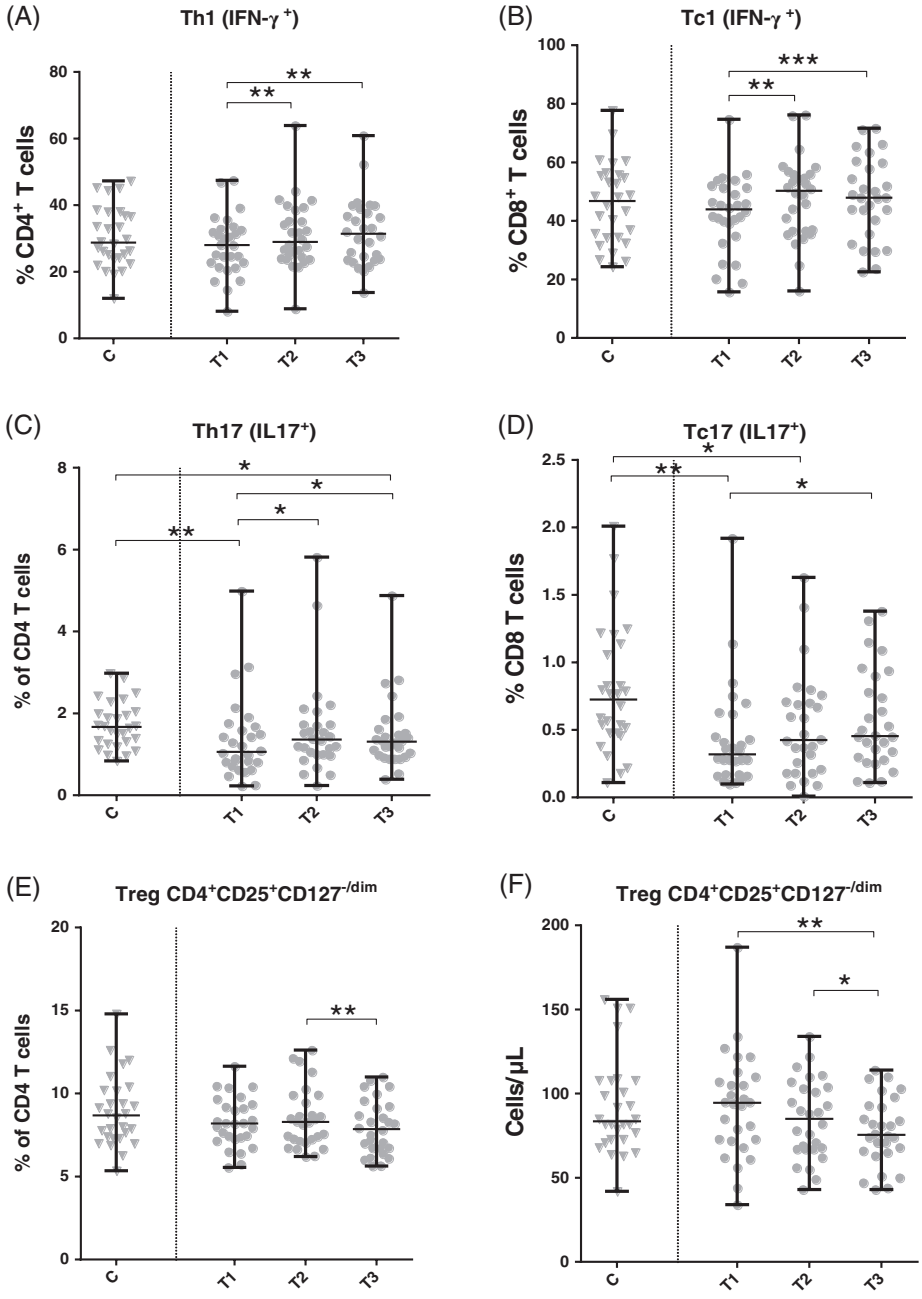

FIGURE 3 T-cell differentiation subsets from T1D onset to established disease. Peripheral pattern of Th1 (A), Tc1 (B), Th17 (C), Tc17 (D) and Treg (E, F) from T1D children and healthy controls. PB collection at disease onset (T1), partial remission (T2) and disease establishment (T3). **P* < .05, ***P* < .01, ****P* < .001 by Mann-Whitney test (patients vs controls) or Wilcoxon matched-pairs signed rank test (comparison within patients in different time-points)

IL17-producing cells are involved in both neutrophil recruitment and activation at inflammatory sites.[25] Similar to other autoimmune diseases, significant migration of Th17 cells and neutrophils to the target organ can play a major role in early disease stages.[26]

Evidence for Th17 cells in the pancreas[27] and pancreatic lymph nodes,[28] as well as the presence of neutrophils in pancreatic tissues of T1D donors, but not of healthy donors,[23] supports the recruitment of neutrophils into inflammatory areas, leading to pancreatic tissue damage.[7] Circulating neutrophil reduction went along with the early phase of active, ongoing and specific autoimmune pancreatic beta-cell destruction.[7] In line with this hypothesis, we observed that neutrophils positively correlated with the duration of remission phase (unpublished results).

In our T1D population, neutrophils started to recover at remission, significantly increasing at established disease. Differently, prolonged mild neutropenia has been described, with later resolution, 5 years after diagnosis.[23] An explanation for these different results might be the relapsing-remitting character of T1D disease course. We speculate that a regain of neutrophils during remission phase results not only from a reduction of migratory events toward the pancreas, but also from increased medullary neutrophil differentiation, promoted by Th17 immunity.[29] A similar pattern was evident in our study for both neutrophils and IL17-producing cells in T1D patients, reinforcing that the Th17/neutrophil pathway is primarily involved in the immunopathology of T1D.[7] Reports on circulating levels of IL-17-producing cells in T1D are controversial, showing either increased[30,31] or unchanged levels compared to controls.[32] It may be argued that lower numbers of peripheral IL-17-producing cells reflect increased recruitment to the target organ and draining lymph nodes that outweighs numbers of circulating cells.[28] In addition, the synergism between Th17 and Th1 cells is generally accepted in T1D pathogenesis.[27] Accordingly, we have identified double positive IL-17$^+$IFN-γ$^+$

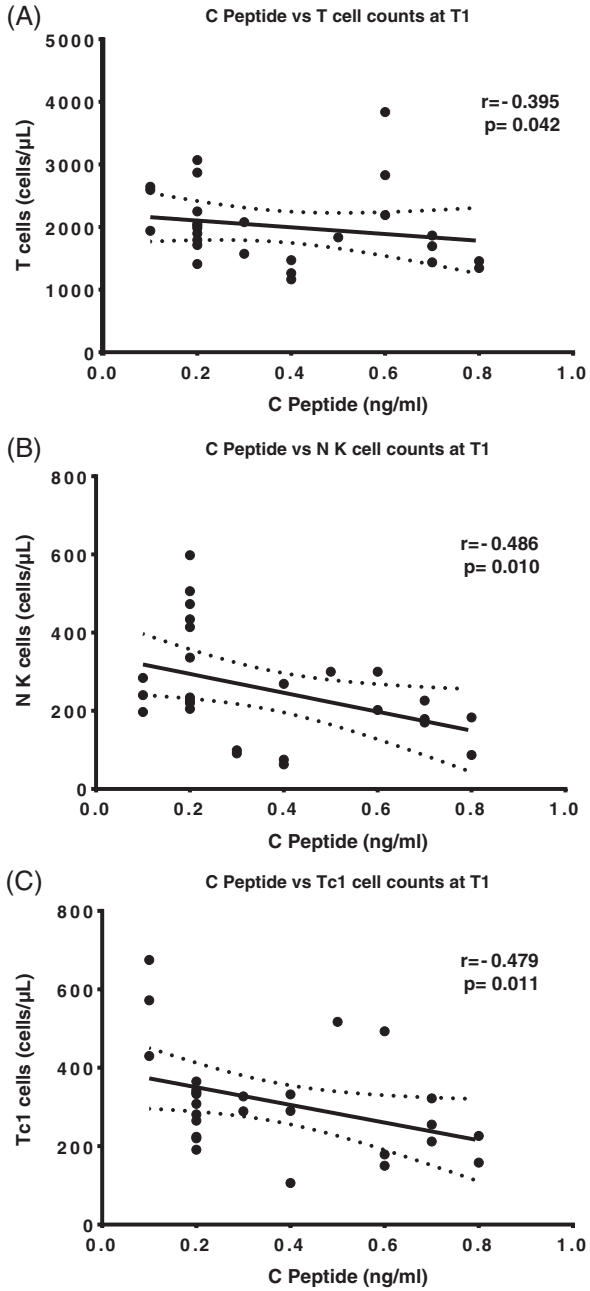

FIGURE 4 Correlation between immune cell populations and fasting C-peptide at T1D onset. T-cell (A), NK (B) and Tc1 (C) absolute counts are shown in correlation to fasting C-peptide. $P < .05$ by Spearman's rank order correlation

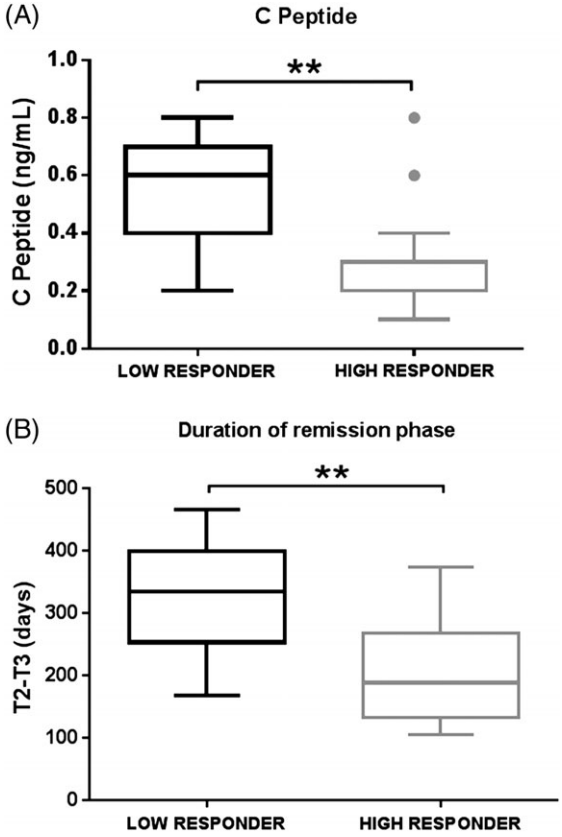

FIGURE 5 Fasting C-peptide levels and remission phase duration in T1D children with low and high-cytokine responses. Fasting C-peptide (A) and duration of remission phase (B) were compared between patients in 2 categories of cytokine response: low responders (LR) and high responders (HR). $^{**}P < .01$ by Mann-Whitney test

secreting T-cells in T1D and controls (unpublished observation), confirming earlier observations.[31]

In accordance with previous studies, Treg counts did not differ between patients and controls.[18,33] However, a Treg depletion has been described in new-onset[34] and long-standing T1D.[34,35] Differences in phenotyping strategies used for Treg identification may explain discrepancies between studies.[30] On the other hand, the significant decrease in Tregs at T3 may reflect a quantitative impairment resulting from long-term activation of these cells during the chronic autoimmune inflammatory process.[36] The increase in Th17/Treg ratio observed during the disease course in our T1D children has been described before.[37] This dynamics highlights again the role of Th17 immunity in T1D,[38,39] sustaining the idea that the classic Th1/Th2 paradigm is not sufficient to explain the immunopathogenic events that lead to autoimmune diabetes.

Though NK frequencies varied in different studies reduced NK counts have been previously described in T1D children.[40] Whether NKs are disease-controlling or disease-promoting cells in T1D is still unclear.[41] However, the fact that T1D children with higher levels of NK-cells have shorter remission phases (unpublished observation) suggests a pathogenic pancreatic involvement of NK-cells in T1D.

Increased circulating cytotoxic cells, namely NK-cells or Tc1 cells, and a higher cytokine response in those children with lower C-peptide levels at disease onset reflects a close relation between immune subsets and metabolic counterparts in T1D children. Therefore, a link between more severe disease patterns and inflammation-prone environment is likely to exist.

Cytokines are known to be involved in autoimmune destruction of beta-cells.[42,43] Other groups have attempted to use single cytokines as disease progression markers, but no correlations were found during the first year of disease.[16] Pro-inflammatory cytokines in our T1D patients were comparable to age-matched healthy controls, similar to recent data on circulating IL-17 in T1D children and controls under 15 years of age[44] as well as to cytokine and chemokine levels between T1D patients and brain-dead organ donors.[45]

Although cytokines are typically classified as pro- or anti-inflammatory, growing evidence suggests that the activity of a given cytokine may be influenced by the timing of response in disease course, its concentration and the co-existing cytokines in the local milieu.[46,47]

Indeed, our results demonstrate the importance of the bulk cytokine milieu. The fact that anti-inflammatory cytokines are typically expressed in conjunction with the pro-inflammatory cytokine TNF-α indicates that this might be a reactive response to counteract pro-inflammatory cytokine toxicity. The level of cytokine response possibly influences the remaining beta-cell reservoir in the first stages of disease as suggested by the observation that children with lower cytokine levels maintain higher C-peptide at onset and have longer remission phases.

Data on insulitis might explain the lower peripheral B-cell counts. In fact, B-cells represent one of the most abundant populations in the islets after CD8+ T-cells,[48,49] being more frequent in the pancreatic inflammatory infiltrate of late T1D,[50] and inversely correlating with insulin-positivity.[48] Recently, a higher prevalence of B-cells was associated with a more pronounced hyperimmune infiltration and a younger age at diagnosis.[49] However, in our study, no age-correlation was found with B-cell counts.

Addressing study design, the purpose of using control samples was to compare children with and without diabetes. Control samples were collected simultaneously to T1, in different months along the 2 years of data collection. No further evaluations were performed in controls, as variations in their immune profile were not expected (controls did not have any immunologic or inflammatory disorder, there was no relevant seasonal clustering, and the study's time-span of circa 1 year, 398 days mean value of monitoring T1D patients, would not significantly account for age-related variations).

However, a larger sample size, including the evaluation of non-remitters, would strengthen the external validity in future studies, and further organ targeted and functional immune subset studies are required, to correlate the peripheral immune variations with the events occurring in the pancreas.

In conclusion, immune cell patterns and cytokine environment are relevant players in the natural course of the disease. The integrated longitudinal picture of T1D progression represents a basis for disease staging and appropriate patient stratification that will enable individualized approaches for resetting the immune system.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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